Characterization of microRNAs as Post-Transcriptional Modulators of DNA Topoisomerase IIα in Etoposide (VP-16) Resistant K562 Cells.

By Evan E. Kania

Research Advisors: Dr. Jack Yalowich & Dr. Terry Elton

Division of Pharmacology

The Ohio State University College of Pharmacy

Columbus, OH USA

May 2019

Abstract

The enzyme DNA topoisomerase IIα (TOP2α) induces covalent complexes with DNA and produces transient double-strand DNA breaks crucial for processes such as replication and normal chromosomal dysjunction at mitosis. TOP2α is an important target for clinically effective anticancer agents, such as etoposide (VP-16), since these drugs stabilize the otherwise short-lived enzyme-DNA covalent complexes, thereby inducing cytotoxic DNA damage. However, the efficacy of these agents is limited by chemoresistance. Our lab has characterized acquired resistance to VP-16 in human leukemia K562 cells. The cloned resistant cell line, K/VP.5, contains reduced levels of TOP2α compared to parental K562 cells. The goal of this project is to test the hypothesis that TOP2α levels are decreased in K/VP.5 cells, in part, through miRNA-mediated mechanisms.

Pooled miRNA qPCR profiling experiments were performed to investigate the expression levels of ~500 miRNAs in K562 and K/VP.5 cells. hsa-miR-9-3p and -5p (miR-9-3p and -5p) were overexpressed in K/VP.5 cells compared to K562 cells. The TOP2α 3′-UTR harbors putative miRNA recognition elements (MRE) for these miRNAs. Therefore, these miRNAs were chosen for further study. To assess post-transcriptional regulation of TOP2α by miRNAs, a dual luciferase reporter plasmid harboring the entire 3′-UTR of TOP2α mRNA (998 bp) was constructed (psiTOP2α/UTR). Transfection with psiTOP2α/UTR demonstrated decreased luciferase expression in K/VP.5 compared with K562 cells, suggesting altered post-transcriptional regulation in resistant cells. K562 cells that were co-transfected with psiTOP2α/UTR and miR-9-3p or -5p mimic resulted in a statistically significant decrease in luciferase expression only for miR-9-5p. Mutating the putative miR-9-5p seed sequence prevented the decrease in luciferase activity, demonstrating a direct interaction of this miRNA

with the MRE of TOP2α. Immunoblotting for TOP2α in K562 cells transfected with miR-9-3p or -5p mimic resulted in decreased TOP2α protein compared to mock transfected K562 cells. Conversely, immunoblotting for TOP2α in K/VP.5 cells transfected with miR-9-3p or -5p inhibitor resulted in an increase of TOP2α protein, strongly suggesting a role for both miRNAs in acquired resistance to VP-16.

Our findings indicate that miR-9-3p and -5p regulate TOP2α expression levels. In addition, results presented here contribute to the elucidation of chemoresistance mechanisms and have the potential for circumvention of drug resistance by modulation of miRNA concentrations.

Introduction

The enzyme human DNA topoisomerase IIα (TOP2α) induces covalent complexes with DNA and produces transient double-strand DNA breaks crucial for processes such as replication and normal chromosomal dysjunction at mitosis (1). The TOP2α enzyme exists as a homodimer whose monomers utilize a tyrosine active site as a nucleophile to begin a reversible transesterification reaction with the 5′-nucleobase scissile sites on the phosphate backbones of the top and bottom strands of DNA (1). This cleavage and subsequent religation of DNA allows for the relaxation of specific DNA entanglements (2-5). Highly proliferative cells are dependent on TOP2α to relieve torsional stress resulting from DNA topological entanglements during replication (6). Hence TOP2α has been utilized as an important target in cancer (1,7-9). Type IIA topoisomerase interfacial inhibitors, such as VP-16, stabilize the otherwise short-lived enzyme-DNA complexes by interjecting themselves between the four base regions of the scissile break sites generated by TOP2α (9,10). This action inhibits the religation of double-strand breaks and the disengagement of TOP2α from DNA. Programmed cell death ensues due to an aggregation of double-strand DNA breaks (9).

Our lab has characterized acquired resistance to VP-16 in human leukemia K562 cells. This cloned resistant cell line, K/VP.5, was found to contain one-fifth the level of TOP2α protein and one-third the level of the corresponding mRNA compared to parental K562 cells (11) (Figure 1). Previously, we showed that one determinant of this observed decrease is due to alternative RNA processing of TOP2α that results in a C-terminal truncated isoform. We also demonstrated that this shortened TOP2α isoform (TOP2α/90) elicits resistance via a dominant negative effect on full-length TOP2α (TOP2α/170) (12). However, alternative RNA processing

did not account for the entire decrease in TOP2α. Thus, other potential determinants of gene regulation were investigated.

MicroRNA (miRNA) mediated post-transcriptional gene silencing is an established mechanism of genetic regulation (13). miRNAs are a family of small non-coding RNAs that are 21-25 nucleotides in length. They originate as precursor pri-miRNAs that are transcribed by RNA polymerase II. During processing, the pri-miRNA is shortened into a miRNA duplex. One strand of the miRNA duplex is selected to be incorporated into the RNA-induced silencing complex (RISC) (14). The miRNA-RISC complex (miRISC) is guided by a seed sequence (spanning from position 2 to 8 at the 5′ end of the miRNA) that base pairs to a specific miRNA recognition element (MRE) typically harbored in the 3′-untranslated region (3′-UTR) of a target mRNA (15,16). MREs are also found in the 5′-untranslated region (5′-UTR) and coding region (CDS) of mRNAs (17-19). Upon binding to the mRNA, miRISC will subsequently reduce the expression of the target mRNA by blocking the ribosome from translating the mRNA or by initiating the degradation of the mRNA. Blocking the ribosome or degrading the mRNA by miRISC is determined by the type of Argonaut (AGO) present in miRISC, the degree of complementarity between the miRNA and mRNA, and the accessibility of the MRE (15,20,21). In the majority of mammalian cells, miRISC serves to block rather than degrade the mRNA (22).

In this study, we have examined the hypothesis that TOP2α expression can be regulated by miRNAs in K562 cells. Given the low expression of TOP2α in K/VP.5 cells, miRNAs overexpressed in K/VP.5 cells with corresponding MREs in the 3′-UTR of TOP2α were of particular interest. Experimental analyses demonstrated that hsa-miR-9-3p and -5p (miR-9-3p and -5p) were overexpressed in K/VP.5 cells and regulated the levels of TOP2α.

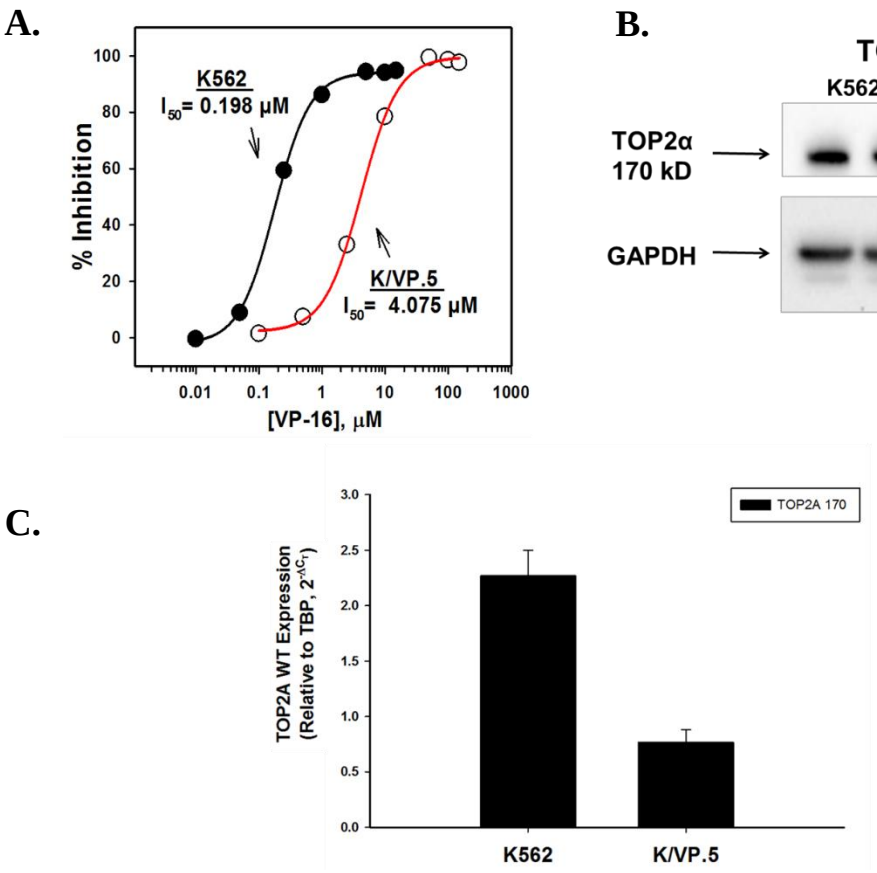


Figure 1. A: K562 cells were ~20-fold resistant to VP-16 compared to parental K562 cells in a 48-hour grown inhibition assay. B-C: The expression levels of TOP2α protein (immunoblot) and mRNA (by qPCR) were attenuated in K/VP.5 cells compared to K562 cells.

Materials and Methods

Plasmids

A 998-bp fragment encoding for the entire TOP2α 3′-UTR was PCR amplified by utilizing sense (5′-AGGCGATCGCTCGAGAATGTGAGGCGATTATTTTAAGTAA-3′) and antisense (5′-ATTCCCGGGCTCGAGTGGGTTGCAATGTTTAGAACATT-3′) primers using standard procedures with a proofreading polymerase, CloneAmp HiFi, from Takara Bio Inc. (cat. No. 639298; Kusatsu, Shiga, Japan). Genomic DNA from K562 cells was used as template. A psiCHECK-2 vector purchased from Promega Corporation (cat. No. C8021; Madison, WI, USA) was linearized with restriction digest enzymes NotI and XhoI from New England Biolabs (cat. No. R0189S, R0146S; Ipswich, MA) according to the manufacturer's protocol. The TOP2α 3′-UTR PCR product was subcloned into the linearized psiCHECK-2 vector utilizing an In-Fusion HD Cloning Kit (cat. No. 638916; Takara Bio Inc.) according to the manufacturer's protocol. The TOP2α 3′-UTR sequence cloned into the multiple cloning site (MCS) of psiCHECK-2 was expressed as fusions to the C-terminus of Renilla luciferase. Plasmid DNA was isolated from recombinant colonies and sequenced to ensure authenticity. The resulting recombinant plasmid was designated psiTOP2α/UTR.

The mutant reporter construct psiTOP2α/UTR-mut-5p was generated utilizing the psiTOP2α/UTR vector as template and mutating the miR-9-5p MRE (located at 703-709 bp) harbored in the TOP2α 3′-UTR using a Q5 Site-Directed Mutagenesis kit from New England Biolabs (cat. No. E0554S) and following the manufacturer's instructions. The primers utilized for mutagenesis were sense (5′-**AAGCGTG**GAGAAACCAATTTCTAAG-3′) and antisense (5′-GAGATTCAGACTCAGAGGCAGC-3′). The nucleotides that were mutated are shown in bold print. The mutation of the miR-9-5p seed sequence was confirmed by dideoxy chain

termination sequencing. The mutant reporter construct psiTOP2α/UTR-mut-3p was generated in the same manner as psiTOP2α/UTR-mut-5p, but at (466-471 bp) with the primers sense (5′-**GAAACT**GGTTCTAGTACAGATAC-3′) and antisense (5′-TTCTATCTGATGGTAAATTATG-3′). Lastly, transformed bacterial cultures were grown and each reporter construct was isolated with a Qiagen Plasmid Midi Kit (cat. No. 12143; Hilden, Germany).

Cell Culture

Human K562 leukemia cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (FS). Cloned etoposide-resistant K562 cells (K/VP.5) were generated by continuous incubation in 0.5 μM etoposide followed by isolation by limiting dilution, as previously described (23). K/VP.5 cells were maintained in DMEM/10% FBS with 0.5 μM etoposide added every other week.

Luciferase Assay

K562 and K/VP.5 cells (in exponential phase growth) were washed and lysed with passive lysis buffer (cat. No. E1941; Promega), and firefly and Renilla luciferase activities were determined by using the Dual-Luciferase Reporter Assay System (cat. No. E1910; Promega) with a Synergy H1 Hybrid Multi-Mode Reader (BioTek, Winooski, VT, USA). Renilla luciferase expression in the psiCHECK-2 vector is generated via an SV40 promoter. Firefly luciferase in this vector is generated via a HSV-TK promoter and has been specifically designed to be an intraplasmid transfection normalization reporter. The Renilla luciferase signal was therefore normalized to the firefly luciferase signal (Scheme 1).

Real-time PCR

Total RNA was isolated from K562 and K/VP.5 cells using PureZOL RNA Isolation Reagent from Bio-Rad Laboratories (cat No. 7326880; Hercules, CA, USA). Digestion of DNA was then performed using TURBO DNAase from Invitrogen (cat. No. AM2238; Carlsbad, CA, USA). Mature human miR-9-3p, -5p, and RNU48 were quantified by utilizing TaqMan assay kits specific for each RNA molecule from Applied Biosysems (cat. No. 002231 (miR-9-3p), 000583 (miR-9-5p), 001006 (RNU48), Foster City, CA, USA). Ten ng of total RNA underwent reverse transcription with miR-9-3p, -5p, and RNU48 antisense primers in a 15 µl reaction according to manufacturer instructions. Quantitative real-time PCR (20 µl reaction) was performed by using 1.33 µl of cDNA with primer/probe sets specific for miR-9-3p, -5p, and RNU48. Gene expression was calculated relative to RNU48.

RNA isolation and DNase treatment for downstream TOP2α mRNA quantification via qPCR was performed as outlined above. Random primed cDNA was synthesized from one µg RNA, according to the manufacturer's protocol, using the High Capacity cDNA Reverse Transcription Kit (cat. No. 4368814; Applied Biosystems). Quantitative real-time PCR (20 µl reaction) was performed by using 5 µl of a 1:5 dilution of cDNA with primer/probe sets specific for TOP2α and TBP (Hs01032136_m1, Hs00427620_m1; Applied Biosystems). Gene expression was calculated relative to TBP.

Western blot analysis

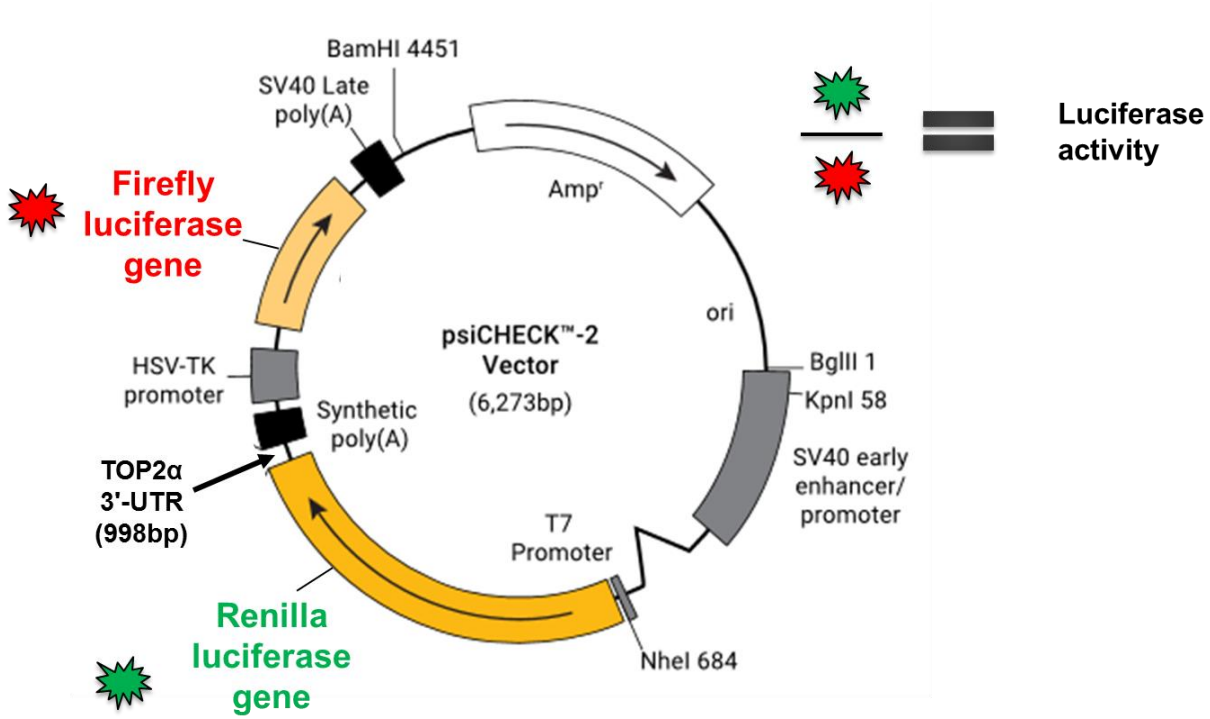
K562 and K/VP.5 cells were pelleted and washed in phosphate-buffered saline. After washing, 1.5 million cells were lysed in 2x Laemmli sample buffer (cat. No. 1610737; Bio-Rad Laboratories) containing 5% β-mercaptoethanol, and boiled at 100 °C for 5 minutes. DNA was

sheared using a sonicator (Tekmar, Mason, OH, USA) at 35 W three times in 10 second intervals. Samples were electrophoresed on a NuPAGE 4-12% Bis-Tris gel from ThermoFisher (cat. No. NP0322BOX; Waltham, MA, USA). Duplicate samples were run with an equal amount of protein (16.6 µg) loaded into each well. Protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) using a semidry electroblotter (ThermoFisher). The PVDF membrane was then sectioned. The 75-250 kDa portion was labeled by overnight incubation at 4 °C with rabbit polyclonal antibodies raised against the N-terminal sequence of human TOP2α (cat. No. ab74715; Abcam, Cambridge, MA; used at 1:1000 dilution). The 25-75 KDa portion was labeled by overnight incubation at 4 °C with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (1:5000; cat. No. sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA). PVDF membranes were subsequently incubated at room temperature for 1.5 hours with a donkey anti-rabbit or donkey anti-mouse secondary antibody respectively (1:8000 dilution) (Jackson ImmunoResearch, West Grove, PA). Antibody-labeled TOP2α and GAPDH were detected using the Immun-Star horseradish peroxidase Chemiluminescence Kit (cat. No. 170-5040; Bio-Rad Laboratories). All immunoassay images were taken with the ChemiDoc XRS+ imaging system and analyzed with ImageLab software (Bio-Rad Laboratories).

Transfection with Mimic and Inhibitors

K562 and K/VP.5 cells (5 x 10^5 in 5 mL per condition) were transfected with dual luciferase plasmids (psiTOP2α/UTR, psiTOP2α/UTR-3pmut, psiTOP2α/UTR-5pmut) utilizing 1 µg of the respective plasmid, 5.5 µl Lipofectamine 3000 (cat. No. L3000008; Invitrogen), and 250 µl Opti-Mem Medium (cat. No. 31985062; Invitrogen). miRNA mimic (cat. No. MIMAT0000441 (miR-9-3p); MIMAT0000442 (miR-9-5p); 4464058 (miRNA mimic negative

control #1); Ambion, Foster City, CA, USA) and inhibitor (MIMAT0014627 (miR-9-3p); MIMAT0036307 (miR-9-5p); 4464076 (miRNA inhibitor negative control #1); Ambion) transfections were similarly performed, but achieving a total concentration of 25 nM of the respective mimic or inhibitor). Forty-eight hours after transfection, cellular extracts were prepared and qPCR, luciferase, and immunoblotting assays were performed as outlined above.



Scheme 1. TOP2α 3′-UTR was inserted into the psiCHECK-2 plasmid containing dual luciferase capability. Renilla luciferase served as the reporter gene regulated by the TOP2α 3′-UTR. Firefly luciferase served as the normalizer gene.

Results

miR-9-3p and -5p are Overexpressed in K/VP.5 Cells

Utilizing the methods reported above, pooled qPCR was performed to investigate differentially expressed miRNAs in K562 versus K/VP.5 cells using total RNA. Over 500 miRNAs were analyzed. The top five miRNAs overexpressed in K/VP.5 cells were hsa-miR-9-5p, hsa-miR-375-3p, hsa-miR-9-3p, hsa-miR-149, and hsa-miR-383 (Fig 2A). This data from the initial pooled qPCR was validated for miR-9-3p and -5p in K/VP.5 cells through qPCR using total RNA (Fig. 2B).

miRNA-mediated genetic regulation results when a miRNA recognizes a specific MRE on a mRNA and subsequently inhibits its translation or initiates its degradation (15,16). Bioinformatics tools have been developed to predict miRNA/mRNA binding (24,25). TargetScan was used to predict putative MREs located in the 3′-UTR of TOP2α and additional MREs were predicted manually. miR-9-3p and -5p were chosen for further study as both had putative MREs in the 3′-UTR of TOP2α, indicating a high probability of binding to TOP2α mRNA versus other miRNAs that were overexpressed in K/VP.5 cells without MREs in the 3′-UTR of TOP2α (15,16). The type of MRE for miR-9-3p and -5p were assessed to aid in determining the likelihood of the predicted MREs as being functionally important in the regulation of TOP2α expression with respect to either miRNA's seed sequence. miR-9-3p had a 6mer seed sequence and miR-9-5p had a more robust 7mer-m8 seed sequence (Fig. 2C). Additionally, a context score was calculated for miR-9-5p to further predict its functionality. A context score is based on MRE site-type, 3′ pairing, local AU effect, position of the 3′-UTR, target site abundance, and seed-pairing stability (24). A context score below -0.30 was used as a cutoff as previously described

(26) and the context score for miR-9-5p was -0.03. No context score was provided for miR-9-3p as its site type was predicted manually.

Differential Regulation of TOP2α 3′-UTR in K562 and K/VP.5 Cells

To investigate if the 3′-UTR of TOP2α is affected differently in K/VP.5 cells compared to K562 cells, a dual luciferase reporter assay was employed. The justification for using this assay is that the binding of a given miRNA to its distinct MRE will repress reporter protein production, thereby reducing luciferase activity. Accordingly, the entire 3′-UTR (998-bp) was subcloned directly downstream of the *hR-luc* open reading frame. This construct was labeled psiTOP2α/UTR and contains the aforementioned MREs for miR-9-3p and -5p. To assess differential regulation of TOP2α 3′-UTR, psiTOP2α/UTR was transfected into K562 and K/VP.5 cells and luciferase activity was measured. Luciferase activity was decreased in K/VP.5 cells compared to K562 cells (Fig. 3). This result suggests that some factor(s) in resistant cells must be acting on the 3′-UTR of TOP2α to cause the observed decrease in luciferase activity.

miR-9-5p Directly Affects TOP2α 3′-UTR

To investigate whether miR-9-3p or -5p can regulate TOP2α expression, the same luciferase reporter assay system was utilized. K562 cells have low amounts of miR-9-3p and-5p (compared to K/VP.5 cells) and are therefore appropriate to study these miRNAs through gain-of-function analyses. K562 cells were co-transfected with psiTOP2α/UTR and either miR-9-3p or -5p mimic. Cells were grown for 48 hours and lysed while in log phase. Additionally, RNA was isolated and qPCR was performed to assure that mimic transfection resulted in miRNA overexpression. Both miR-9-3p and -5p levels increased significantly in K562 cells ($P<0.05$) when transfected with the respective mimic with no cross-over effect (no increase in the non-

transfected mimic level) (Fig. 4A). Luciferase activities were subsequently measured (Fig. 4B). These experiments demonstrated that a statistically significant decrease ($P<0.05$) in luciferase activity resulted when K562 cells were transfected with miR-9-5p. Luciferase activity was not decreased significantly upon transfection with miR-9-3p.

To validate that miR-9-5p interacted with the predicted MRE in the 3′-UTR of TOP2α, an additional luciferase reporter construct was generated in which the entire 7-bp MRE (CCAAAGA), specific to the 5′ seed sequence of miR-9-5p, was mutated (AAGCGUG). The mutant construct was labeled psiTOP2α/UTR-mut-5p. Subsequent co-transfection of K562 cells with psiTOP2α/UTR-mut-5p and miR-9-5p mimic resulted in no significant difference in luciferase activity between the two conditions (Fig. 4C). These results support direct binding of miR-9-5p to the MRE on the 3'UTR of TOP2α. For completeness, another luciferase reporter construct was generated in which the entire 6-bp MRE (CUUUAU), specific to the 5′ seed sequence of miR-9-3p, was mutated (GAAACU). The mutant construct was labeled psiTOP2α/UTR-mut-3p. Subsequent co-transfection of K562 cells with psiTOP2α/UTR-mut-3p and miR-9-3p mimic resulted in no significant difference in luciferase activity between the two conditions (Fig. 4D). However, since miR-9-3p did not statistically significantly decrease luciferase activity of psiTOP2α/UTR in K562 cells (Fig. 4B), it is not likely that miR-9-3p binds directly to its putative MRE in the 3′-UTR of TOP2α.

miR-9-3p and -5p Lower Expression of TOP2α in K562 Cells

If TOP2α mRNA is an authentic target of miR-9-3p or -5p, then manipulation of the endogenous expression of miR-9-3p or -5p should result in predictable changes in TOP2α protein levels. For instance, a gain of function analysis through overexpression of a given miRNA should lead to a decrease in target protein expression. Conversely, a loss of function

analysis through transfection of a given miRNA inhibitor should lead to greater target protein expression; via decreased translational repression or mRNA degradation due to an inhibition of the function of specific mature miRNAs. In this study, gain-of-function experiments were performed in K562 cells (low miR-9 levels) to evaluate effects of overexpression of miR-9-5p and miR-9-3p on TOP2α protein expression. Conversely, loss-of-function experiments were performed in K/VP.5 cells (high miR-9 levels) to evaluate effects of forced expression of inhibitors of miR-9-5p and miR-9-3p on TOP2α protein expression.

Gain-of-function experiments were performed in K562 cells by transfecting these cells with 25 nM miR-9-3p or -5p mimic, and TOP2α levels were subsequently measured by immunoblotting assays. TOP2α levels in cells transfected with miR-9-3p or -5p mimic resulted in statistically significant decreases (51.7% and 60.4% respectively) compared to control ($P<0.05$) (Fig. 5A).

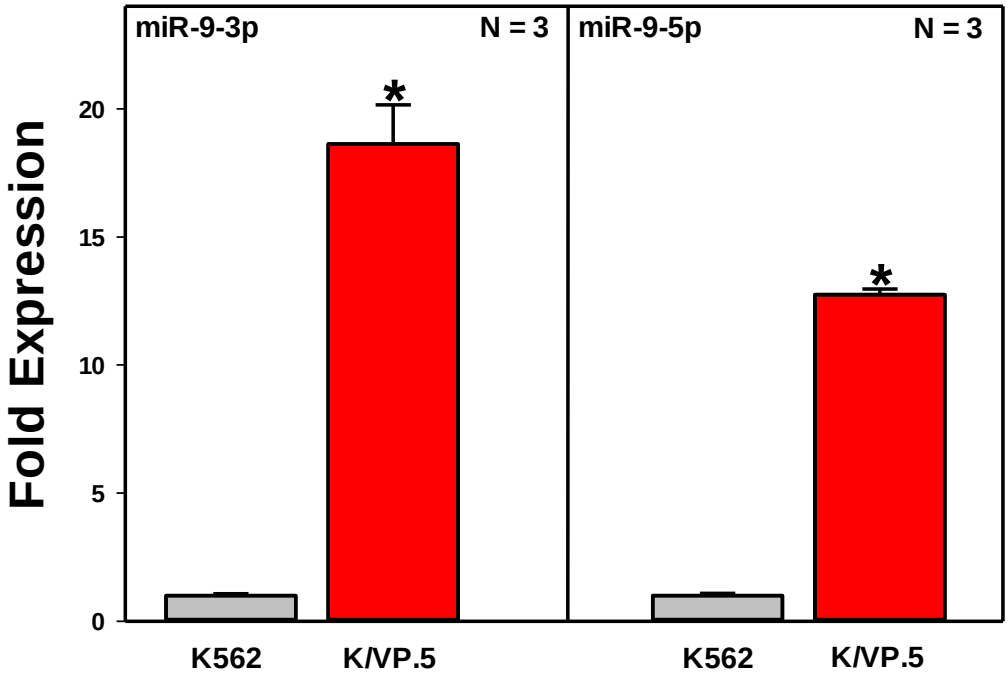
Loss-of function experiments were performed in K/VP.5 cells by transfecting these cells with 25 nM miR-9-3p or -5p inhibitor, and TOP2α levels were subsequently measured by immunoblotting assays. TOP2α levels in cells transfected with miR-9-3p or -5p inhibitor resulted in statistically significant increases (239.3% and 255.3% respectively) compared to control ($P<0.05$) (Fig 5B).

The potential mechanism by which miR-9-3p and -5p reduces TOP2α levels was assessed by real-time qPCR. RNA isolated from K562 and K/VP.5 cells, transfected with mimic or inhibitor, respectively, was assayed for TOP2α mRNA. Transfection with miR-9-3p or -5p mimics or inhibitors did not decrease TOP2α steady state mRNA levels (Fig. 5C) suggesting that miR-9 posttranscriptional effects are at the level of translation as is reported in the literature for miRNAs in general (22), rather than by perturbing mRNA.

A.

Sanger Name	Fold Expression (K/VP.5)/(K562)	Seed Sequence in 3′UTR of TOP2α
hsa-miR-9-5p	22.4	**YES**
hsa-miR-375-3p	14.3	NO
hsa-miR-9-3p	13.1	**YES**
hsa-miR-149	12.9	NO
hsa-miR-383	11.3	NO

B.



C.

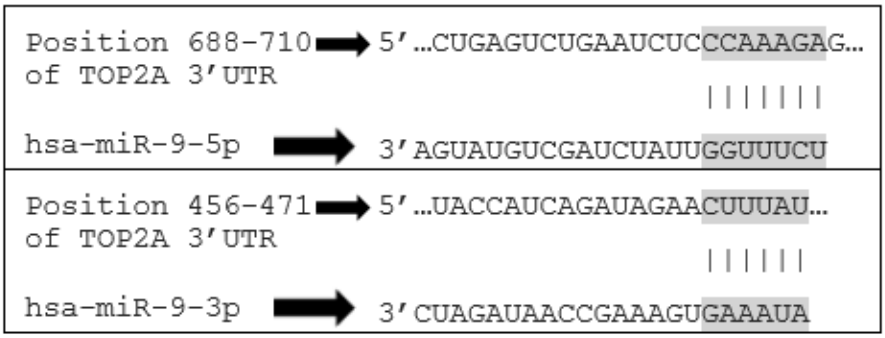


Figure 2. A: miRNAs are overexpressed in K/VP.5 cells compared to K562 cells. B: miR-9-3p and -5p overexpression in K/VP.5 cells compared to K562 cells was validated. C: miR-9-3p and -5p have seed sequences that correspond to MREs in the 3′-UTR of TOP2α.

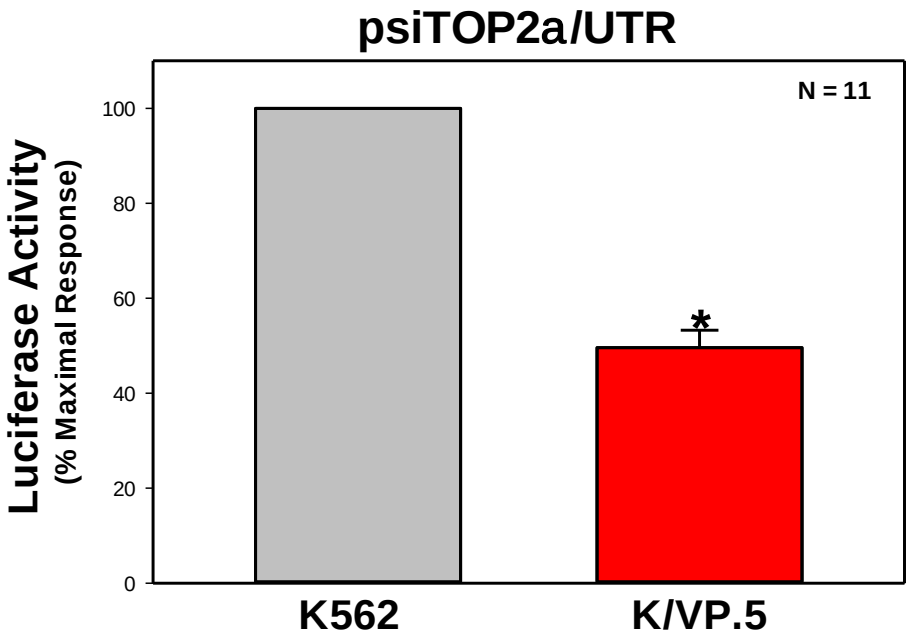


Figure 3. Luciferase activity generated from the psiTOP2α/UTR vector was reduced in K/VP.5 cells compared to K562 cells.

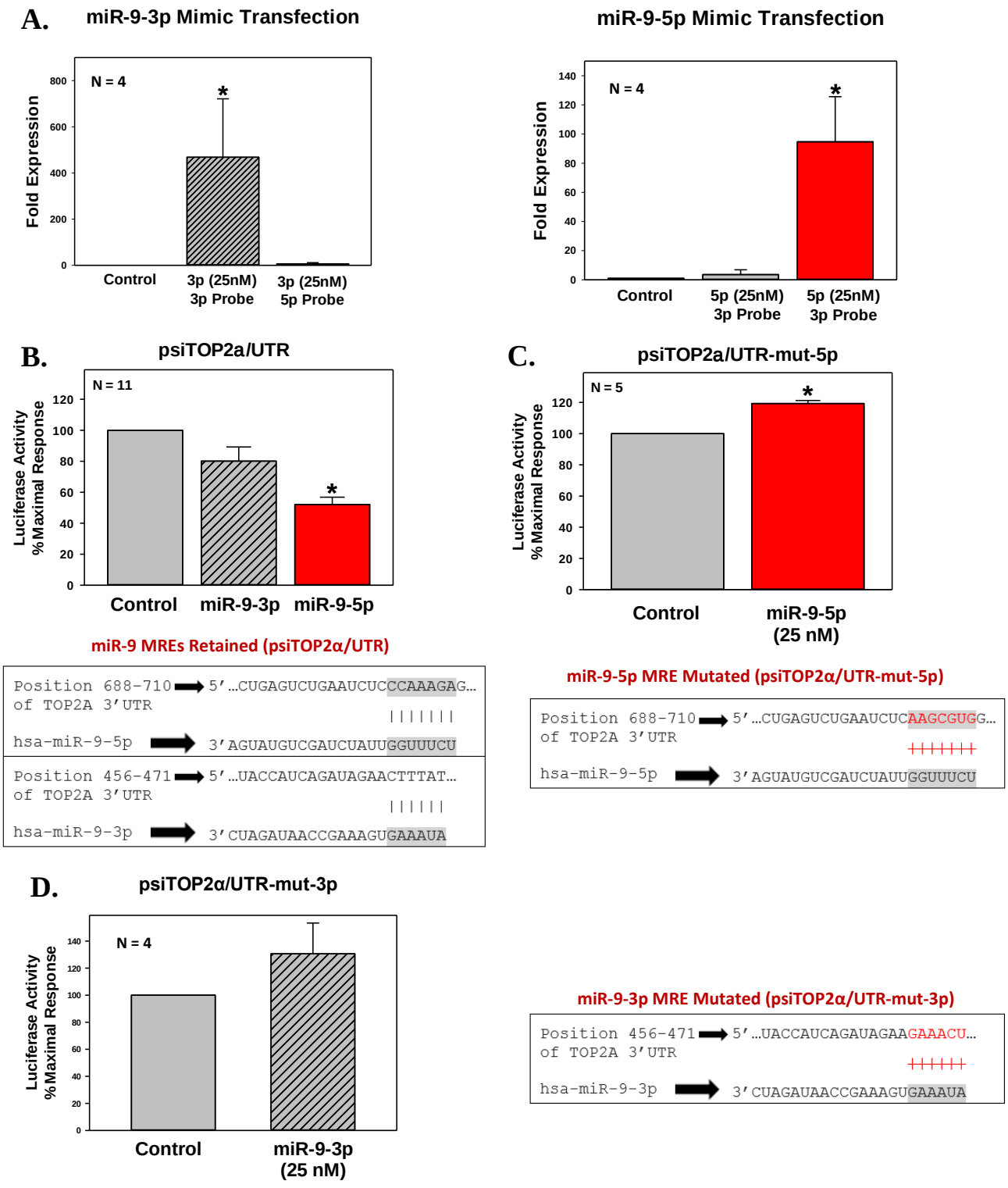


Figure 4. A: Transfection of miR-9-3p or –5p mimics resulted in overexpression of these miRNAs in K562 cells. B: Transfection of K562 cells with miR-9-3p or miR-9-5p mimic resulted in decreased luciferase activity for psiTOP2α/UTR only with miR-9-5p mimic. C: miR-9-5p mimic had no effect when the MRE for miR-9-5p was mutated. D: miR-9-3p mimic had no effect when the MRE for miR-9-3p was mutated.

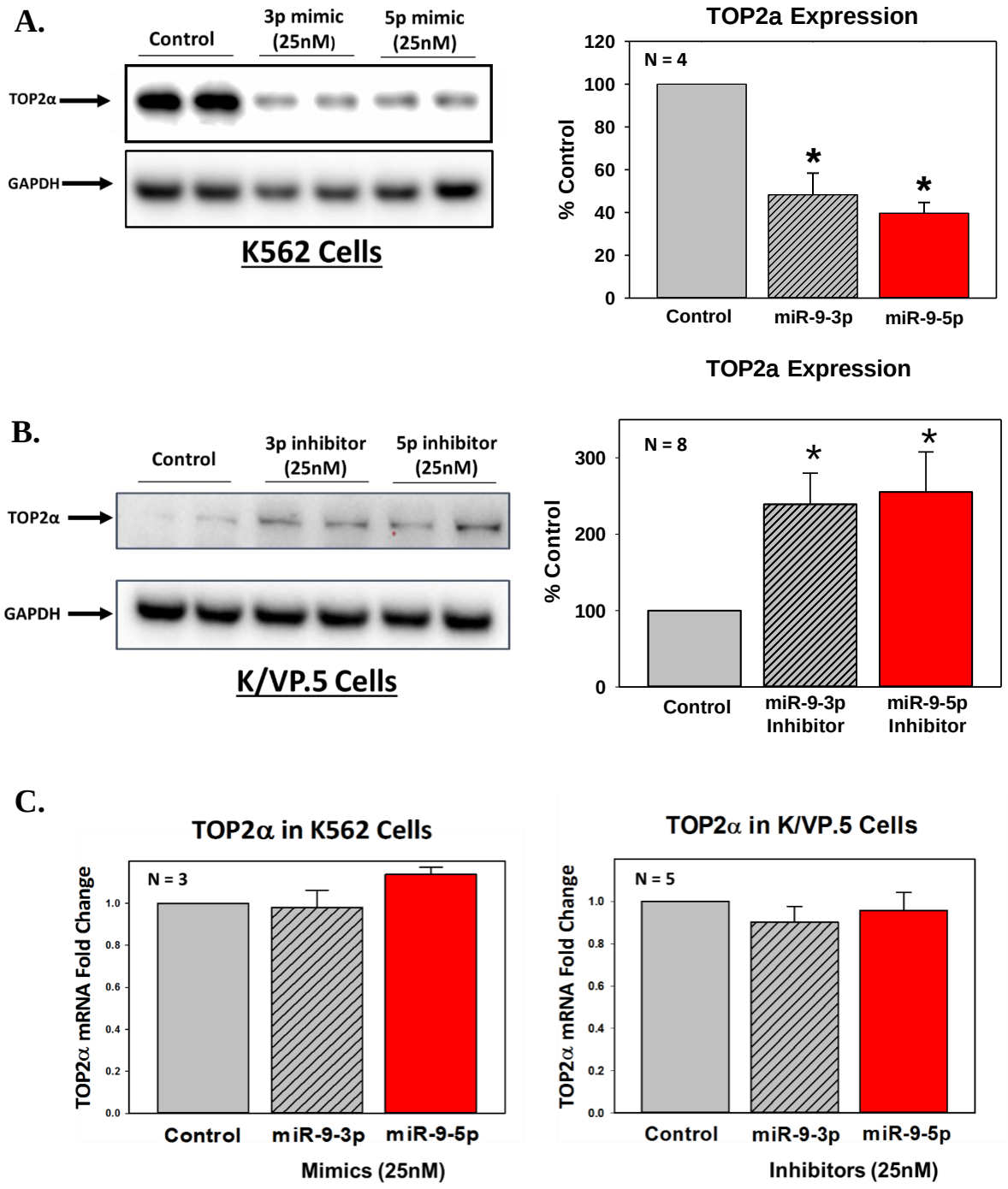


Figure 5. A: miR-9-3p overexpression in K562 cells resulted in a 51.7% decrease in TOP2α. miR-9-5p overexpression in K562 cells resulted in a 60.4% decrease in TOP2α. B: miR-9-3p inhibition in K/VP.5 cells resulted in a 239.3% increase in TOP2α. miR-9-5p inhibition in K/VP.5 cells resulted in a 255.3% increase in TOP2α. C: Forced expression of miR-9-3p or -5p mimics in K562 cells did not affect TOP2α expression. Forced expression of miR-9-3p or -5p inhibitors in K/VP.5 cells did not significantly affect TOP2α mRNA expression.

Discussion and Conclusion

miRNAs are estimated to regulate more than 60% of protein-coding genes in humans post-transcriptionally through binding to specific MREs typically harbored in the 3′-UTR of a target mRNA (13). Due to their importance in gene regulation, dysregulation of miRNAs can have an impact on a variety of physiological functions including cell proliferation, apoptosis, development, and metabolism (27). Specifically, miR-9-3p and -5p are implicated in a plethora of disease states (28-31). In cancer, miR-9-3p and -5p have been shown to have diverse roles by acting as either tumor suppressors by targeting oncogenes or as oncogenic molecules by targeting tumor suppressors (32). For example, it was found that miR-9-5p acts as a tumor suppressor in papillary thyroid cancer by targeting BRAF; malignant tumors had low miR-9-5p and high BRAF whereas nonmalignant tumors had the opposite (33). As an oncogenic molecule, in small cell lung cancer miR-9-5p was found to promote cell growth and metastasis by repression of TGFBR2 (34). Similar to the diverse functions of miR-9-5p, miR-9-3p was found to have tumor suppressor activity in hepatocellular carcinoma through targeting the TAZ oncogene (35). Conversely, upregulated miR-9-3p in medullary thyroid cancer was found to increase cell growth and inhibit apoptosis through targeting BLCAP (36).

Dysregulation of miRNAs in cancer can also impact drug efficacy. For example, the abundant expression of miR-21 in cisplatin resistant epithelial ovarian cancer was found to increase chemoresistance through targeting PTEN (37). Acquired chemoresistance to drugs such as etoposide, amsacrine, doxorubicin, and mitoxantrone is partially characterized by decreased TOP2α expression (38,39). In this report, we have found that there is an abundant expression of miR-9-3p and -5p in etoposide resistant K562 cells (K/VP.5) compared to parental K562 cells

and that they target and decrease the expression of TOP2α, which can contribute to the resistance phenotype.

Previously, we reported that K/VP.5 cells with acquired resistance to etoposide, have reduced levels of TOP2α and an increase, as a result of alternative RNA processing, of a 90 kDa isoform of TOP2α (TOP2α/90) which heterodimerizes with full-length 170 kDa TOP2α (TOP2α/170) and is a determinant of drug resistance (12). Interestingly, there are novel MREs in the truncated TOP2α/90 isoform due to the retention of the TOP2α intron 19 (not shown). These unique MREs might be targeted by miRNAs more abundantly expressed in sensitive K562 cells where the 90 kd TOP2α isoform is less expressed (~3-fold less) compared to the resistant K/VP.5 cells. Investigation of miRNAs that target the 90 kDa TOP2α might further elucidate the role of miRNAs in resistance and therefore merits further study. Additionally, TOP2β is less expressed in K/VP.5 cells compared to K562 cells (11). TOP2β is also a target of VP-16 and its expression has been linked to the efficacy of VP-16's genotoxic effects (40). Therefore, its reduction might contribute to chemoresistance. Preliminary immunoblot data suggests that TOP2β expression is regulated by miR-9-3p and -5p (data not shown). Investigations are underway to examine whether this effect is indirect or direct.

A miRNA's ability to regulate gene expression post-transcriptionally is considered direct if it binds to an MRE on the target mRNA. Indirect effects occur by the miRNA targeting other gene(s) that impact the downstream expression of the gene being studied (41,42). Primarily, the 3′-UTR is targeted by miRNAs, however, there is growing evidence that the 5′-UTR and CDS can harbor functional MREs as well (22,43). Interestingly, TOP2α mRNA does harbor miR-9-3p MREs in the CDS. Studies are ongoing to examine if miR-9-3p is capable of binding to these MREs to inhibit translation. Additionally, AGO HITS-CLIP assays, a recent method of mapping

MREs, has found that up to 80% of miRNA-mRNA binding *in vivo* is mediated through non-canonical MREs (44). A canonical miRNA-mRNA interaction consists of the miRNA's seed region (position 2-8) fully binding to the MRE in a mRNA. Non-canonical binding, that has been documented thus far, occurs when there are mismatches between the miRNA's seed region and the MRE, known as a seed-like motif, or when a guanine at position 6 of the MRE on the mRNA bulges outward and allows the miRNA to fully bind, known as a nucleation bulge (45,46). The ability of miR-9-3p to downregulate TOP2α expression could also be attributed to non-canonical binding. Analysis of TOP2α 3′-UTR for these sites, as well as an AGO HITS-CLIP assay using K562 and K/VP.5 cells are planned.

The major findings in the present study are that miR-9-3p and -5p are overexpressed in K/VP.5 cells and that these miRNAs regulate TOP2α expression. miR-9-5p was found to directly interact with a specific MRE in the 3′-UTR of TOP2α. Yet miR-9-3p was found to not interact at its predicted MRE, suggesting that miR-9-3p regulates TOP2α expression through indirect effects, an MRE in the CDS, or by non-canonical binding. Additionally, other newly discovered miRNAs might be overexpressed in K/VP.5 cells compared to K562 cells. These miRNAs could be playing a role in TOP2α expression and a robust miRNA-seq analysis is underway to examine the constellation of miRNAs that were previously unidentified when pooled miRNA qPCR analysis was performed.

In conclusion, many studies demonstrate the importance of TOP2α as a target of chemotherapies. Our lab previously demonstrated that a decrease in TOP2α through alternative RNA processing was a mechanism of drug resistance (35). However, production of a truncated TOP2α/90 isoform alone did not seem to fully account for the decrease in full-length TOP2α/170 and drug resistance observed.

Given that the expression levels of TOP2α are important for the efficacy of topoisomerase II-targeted drugs, it is necessary to understand the mechanisms by which TOP2α expression is regulated especially in acquired drug resistance. Here, experimental analyses demonstrated that miR-9-3p and -5p are overexpressed in etoposide resistant K562 cells and regulate the expression of TOP2α likely through inhibition of translation. Future studies will begin to investigate the functional role of miR-9-3p and -5p in chemoresistance through DNA damage, apoptosis, and cytotoxicity assays to elucidate the role of miR-9-3p and -5p in etoposide resistance and assess their potential as drug targets and/or biomarkers of chemoresistance.

References

(1) Deweese JE, Osheroff N. The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing. Nucleic Acids Res 2009 Feb;37(3):738-748.

(2) Vos SM, Tretter EM, Schmidt BH, Berger JM. All tangled up: how cells direct, manage and exploit topoisomerase function. Nat Rev Mol Cell Biol 2011 Nov 23;12(12):827-841.

(3) Chen SH, Chan NL, Hsieh TS. New mechanistic and functional insights into DNA topoisomerases. Annu Rev Biochem 2013;82:139-170.

(4) Pendleton M, Lindsey RH,Jr, Felix CA, Grimwade D, Osheroff N. Topoisomerase II and leukemia. Ann N Y Acad Sci 2014 Mar;1310:98-110.

(5) Ashour ME, Atteya R, El-Khamisy SF. Topoisomerase-mediated chromosomal break repair: an emerging player in many games. Nat Rev Cancer 2015 Mar;15(3):137-151.

(6) Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth Differ 1991 Apr;2(4):209-214.

(7) Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. Pharmacol Ther 2003 Aug;99(2):167-181.

(8) Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009 May;9(5):338-350.

(9) Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem Biol 2010 May 28;17(5):421-433.

(10) Laponogov I, Pan XS, Veselkov DA, McAuley KE, Fisher LM, Sanderson MR. Structural basis of gate-DNA breakage and resealing by type II topoisomerases. PLoS One 2010 Jun 28;5(6):e11338.

(11) Kanagasabai R, Serdar L, Karmahapatra S, Kientz CA, Ellis J, Ritke MK, et al. Alternative RNA Processing of Topoisomerase IIalpha in Etoposide-Resistant Human Leukemia K562 Cells: Intron Retention Results in a Novel C-Terminal Truncated 90-kDa Isoform. J Pharmacol Exp Ther 2017 Jan;360(1):152-163.

(12) Kanagasabai R, Karmahapatra S, Kientz CA, Yu Y, Hernandez VA, Kania EE, et al. The Novel C-terminal Truncated 90-kDa Isoform of Topoisomerase IIalpha (TOP2alpha/90) Is a Determinant of Etoposide Resistance in K562 Leukemia Cells via Heterodimerization with the TOP2alpha/170 Isoform. Mol Pharmacol 2018 May;93(5):515-525.

(13) Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009 Jan;19(1):92-105.

(14) Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 2005 Aug 4;436(7051):740-744.

(15) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009 Jan 23;136(2):215-233.

(16) Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol 2007;23:175-205.

(17) Forman JJ, Coller HA. The code within the code: microRNAs target coding regions. Cell Cycle 2010 Apr 15;9(8):1533-1541.

(18) Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 2007 Jun 5;104(23):9667-9672.

(19) Fang Z, Rajewsky N. The impact of miRNA target sites in coding sequences and in 3'UTRs. PLoS One 2011 Mar 22;6(3):e18067.

(20) Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol 2012 Jun 5;19(6):586-593.

(21) Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, et al. Identification of novel argonaute-associated proteins. Curr Biol 2005 Dec 6;15(23):2149-2155.

(22) Catalanotto C, Cogoni C, Zardo G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. Int J Mol Sci 2016 Oct 13;17(10):10.3390/ijms17101712.

(23) Ritke MK, Yalowich JC. Altered gene expression in human leukemia K562 cells selected for resistance to etoposide. Biochem Pharmacol 1993 Dec 3;46(11):2007-2020.

(24) Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife 2015 Aug 12;4:10.7554/eLife.05005. eCollection 2015.

(25) Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res 2013 Jul;41(Web Server issue):169.

(26) Sansom SE, Nuovo GJ, Martin MM, Kotha SR, Parinandi NL, Elton TS. miR-802 regulates human angiotensin II type 1 receptor expression in intestinal epithelial C2BBe1 cells. Am J Physiol Gastrointest Liver Physiol 2010 Sep;299(3):632.

(27) Ardekani AM, Naeini MM. The Role of MicroRNAs in Human Diseases. Avicenna J Med Biotechnol 2010;2(4):161-179.

(28) Yang L, Mu Y, Cui H, Liang Y, Su X. MiR-9-3p augments apoptosis induced by H2O2 through down regulation of Herpud1 in glioma. PLoS One 2017 Apr 21;12(4):e0174839.

(29) Sun YN, Li S, Zhou YT, Liu J, Tian LB, Zhen YF, et al. Inhibition of microRNA-9-3p reduces lipid accumulation in HepG2 cells by targeting the expression of sirtuin type 1. Mol Med Rep 2015 Nov;12(5):7742-7748.

(30) Wang Z, Sun L, Jia K, Wang H, Wang X. miR-9-5p modulates the progression of Parkinson's disease by targeting SIRT1. Neurosci Lett 2019 Feb 28;701:226-233.

(31) Majd M, Hosseini A, Ghaedi K, Kiani-Esfahani A, Tanhaei S, Shiralian-Esfahani H, et al. MiR-9-5p and miR-106a-5p dysregulated in CD4(+) T-cells of multiple sclerosis patients and targeted essential factors of T helper17/regulatory T-cells differentiation. Iran J Basic Med Sci 2018 Mar;21(3):277-283.

(32) Nowek K, Wiemer EAC, Jongen-Lavrencic M. The versatile nature of miR-9/9(*) in human cancer. Oncotarget 2018 Apr 17;9(29):20838-20854.

(33) Guo F, Hou X, Sun Q. MicroRNA-9-5p functions as a tumor suppressor in papillary thyroid cancer via targeting BRAF. Oncol Lett 2018 Nov;16(5):6815-6821.

(34) Li G, Wu F, Yang H, Deng X, Yuan Y. MiR-9-5p promotes cell growth and metastasis in non-small cell lung cancer through the repression of TGFBR2. Biomed Pharmacother 2017 Dec;96:1170-1178.

(35) Higashi T, Hayashi H, Ishimoto T, Takeyama H, Kaida T, Arima K, et al. miR-9-3p plays a tumour-suppressor role by targeting TAZ (WWTR1) in hepatocellular carcinoma cells. Br J Cancer 2015 Jul 14;113(2):252-258.

(36) Chen Y, Zhang S, Zhao R, Zhao Q, Zhang T. Upregulated miR-9-3p Promotes Cell Growth and Inhibits Apoptosis in Medullary Thyroid Carcinoma by Targeting BLCAP. Oncol Res 2017 Sep 21;25(8):1215-1222.

(37) Yu X, Chen Y, Tian R, Li J, Li H, Lv T, et al. miRNA-21 enhances chemoresistance to cisplatin in epithelial ovarian cancer by negatively regulating PTEN. Oncol Lett 2017 Aug;14(2):1807-1810.

(38) Chen AY, Liu LF. Mechanisms of resistance to topoisomerase inhibitors. Cancer Treat Res 1994;73:263-281.

(39) Ganapathi RN, Ganapathi MK. Mechanisms regulating resistance to inhibitors of topoisomerase II. Front Pharmacol 2013 Aug 1;4:89.

(40) Cowell IG, Sondka Z, Smith K, Lee KC, Manville CM, Sidorczuk-Lesthuruge M, et al. Model for MLL translocations in therapy-related leukemia involving topoisomerase IIbeta-mediated DNA strand breaks and gene proximity. Proc Natl Acad Sci U S A 2012 Jun 5;109(23):8989-8994.

(41) Shahab SW, Matyunina LV, Hill CG, Wang L, Mezencev R, Walker LD, et al. The effects of MicroRNA transfections on global patterns of gene expression in ovarian cancer cells are functionally coordinated. BMC Med Genomics 2012 Aug 1;5:33.

(42) Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008 Sep 4;455(7209):64-71.

(43) Hausser J, Syed AP, Bilen B, Zavolan M. Analysis of CDS-located miRNA target sites suggests that they can effectively inhibit translation. Genome Res 2013 Apr;23(4):604-615.

(44) Seok H, Ham J, Jang ES, Chi SW. MicroRNA Target Recognition: Insights from Transcriptome-Wide Non-Canonical Interactions. Mol Cells 2016 May 31;39(5):375-381.

(45) Chi SW, Hannon GJ, Darnell RB. An alternative mode of microRNA target recognition. Nat Struct Mol Biol 2012 Feb 12;19(3):321-327.

(46) Loeb GB, Khan AA, Canner D, Hiatt JB, Shendure J, Darnell RB, et al. Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. Mol Cell 2012 Dec 14;48(5):760-770.

Acknowledgements

Thank you to all the members of the Yalowich-Elton lab for your support and guidance on these projects, especially to my mentors Dr. Jack Yalowich and Dr. Terry Elton. My research experience was supported by the Patrick and Jane O'Neill Endowed Scholarship, a Summer Undergraduate Research Fellowship, an Honors and Scholars Enrichment Grant, and a Research Scholar Award. The research in the Yalowich-Elton lab was supported by an R01 grant, CA226906, from the National Cancer Institute.